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Toward Functional Genomics of Plant-Pathogen Interactions: Isolation and Analysis of Defense-related Genes of Hot Pepper Expressed During Resistance Against Pathogen

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To understand plant-pathogen interactions, a complete set of hot pepper genes differentially expressed against pathogen attack was isolated. As an initial step, hundreds of differentially expressed cDNAs were isolated from hot pepper leaves showing non-host resistance against bacterial plant pathogens (*Xanthomonas campestris* pv. *glycines* and *Pseudomonas syringae* pv. *syringae*) using differential display reverse transcription polymerase chain reaction (DDRT-PCR) technique. Reverse Northern and Northern blot analyses revealed that 50% of those genes were differentially expressed in pepper leaves during non-host resistance response. Among them, independent genes without redundancy were micro-arrayed for further analysis. Random EST sequence database were also generated from various cDNA libraries including pepper tissue specific libraries and leaves showing non-host hypersensitive response against *X. campestris* pv. *glycines*. As a primary stage, thousands of cDNA clones were sequenced and EST data were analyzed. These clones are being spotted on glass slide to study the expression profiling. Results of this study may further broaden knowledge on plant-pathogen interactions.

Keywords : *Capsicum annuum*, cDNA micro-array, DDRT-PCR, EST, functional genomics.

As a sessile organism, plants defend themselves against invading pathogens by exerting diverse cellular responses. The most distinctive phenotype of defense response is the rapid cell death of plant at the site of infection, so called hypersensitive response (HR), to limit spreading of the pathogen (Dangle et al., 1996). In the cellular level, HR accompanies a large set of defense responses, including generation of reactive oxygen species (ROS) (Levine et al., 1994; Mehdy, 1994), cell wall lignification (Whetten and Sederoff, 1995), and biosynthesis of antibiotics (Darvill and Albersheim, 1984; Dixon, 1986). In addition, increased transcriptional levels of defense-related genes, such as PR (pathogen related protein), chitinase, SAR8.2, glucanase, thionin, ubiquitin, catalase, glutathione-S-transferase, cytochrome P450, and 14-3-3 protein, are easily shown (Becker et al., 2000; Lee et al., 2001; Levine et al., 1994; Oh et al., 1999; Roberts and Bowles, 1999; Ward et al., 1991; Whitbred and Schuler 2000; Wu et al., 1999).

Furthermore resistance responses develop in unaffected parts of the plant, SAR (systemic acquired resistance), which provide pre-formed resistance against further infection with a broad spectrum of pathogens (Ryals et al., 1996).

Plant defense responses are orchestrated consequences of transcriptional activation of defense-related genes (Lamb et al., 1989). In the process of local and systemic responses, a large group of pathogenesis-related (PR) proteins are synthesized to display a broad spectrum of anti-microbial activity (Bowles, 1990). In addition to the genes directly related to defense responses such as PR-genes, transcription of the genes encoding enzymes involved in secondary metabolic pathways are stimulated. The most intensively studied secondary metabolisms in this regard are terpenoid and phenylpropanoid pathways for producing phytoalexins and phenolics (1990, Choi et al., 1992 and 1994; Dixon and Lamb). Since the secondary metabolism cannot occur without related primary metabolism where large carbon fluxes are supplied, genes involved in primary metabolisms

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are also expressed. Similarly, genes for the activated methyl cycle have elevated transcriptional activity, possibly to provide the activated methyl groups to be used in ethylene production and numerous methylation steps for secondary product formation (Kawalleck et al., 1992).

The complexity of the plant defense mechanisms is becoming apparent, since pathogen defense entails a major shift in metabolic activity rather than altered expression of a few classes of defense-related genes (Somssich and Hahlbrock, 1998). Therefore, identification of a complete set of genes involved in the defense process is an essential step toward understanding the whole scheme of plant defense mechanisms. In this regard, as recently revealed by genetic studies with *Arabidopsis*, more functionally unidentified plant genes must exist whose products are also required for mounting effective defense responses (Glazebrook, et al., 1996; Rogers and Ausubel, 1997).

Subtractive hybridization, differential screening, differential-display PCR analysis, random expressed sequence tag (EST) sequencing, and micro-array have been developed to isolate differentially expressed genes in organisms (Liang and Pardee, 1992; Kouchi and Hata, 1993; Schena et al., 1995; Velculescu et al., 1995). Differential display is a simple and highly sensitive method to detect mRNAs of low abundance. EST sequencing is a good tool to randomly isolate a number of genes related to a specific condition/tissue. In contrast, micro-array is a relatively new technique and a powerful tool in genomics. Although the use of micro-array is relatively more costly compared with that of other methods, it can generate lots of parallel data.

To contribute to the understanding of plant-pathogen interaction, experiments were performed for the isolation of a mass of genes expressed during plant defense responses. The isolated genes were classified into previously identified defense-related genes, genes encoding primary or secondary metabolic enzymes of known function, and novel genes. Expression patterns of isolated defense-related genes and computational analysis of thousands of EST clones isolated from pathogen-induced pepper cDNA library will be a good starting point toward understanding the complexity of plant defense mechanism and function of defense-related genes.

The Patho System

For the isolation of pathogen defense genes of hot pepper, non-host resistance of pepper plant was used against soybean pustule pathogen, *X. c.* pv. *glycines*. Upon infiltration of this bacterium into hot pepper, the leaf tissues underwent cell death and ended up with typical hypersensitive lesion within 20 h after inoculation (Fig. 1). The symptoms were almost identical with that caused by infection with its own incompatible pathogen such as *X. c.* pv. *vesicatoria* (Lee, S. J. and Oh, S. K., personal communication). Electron micrographic study revealed the dramatic microscopic level difference in pepper-*Xcg* interaction between HR-causing *Xcg* 8ra and HR-mutant of the same pathogen *Xcg* 8-13 (Fig. 1).

Isolation of Defense-Related Genes from Hot Pepper Using Differential Display

In the authors previous studies on tobacco, it was presumed that host and non-host HR in plant probably share signaling pathway(s) leading to hypersensitive cell death (Oh et al., unpublished data). For that reason, non-host resistance of hot pepper was used against soybean pustule pathogen for isolation of mass of genes expressed during HR cell death following infection. From DDRT-PCR procedures, hundreds

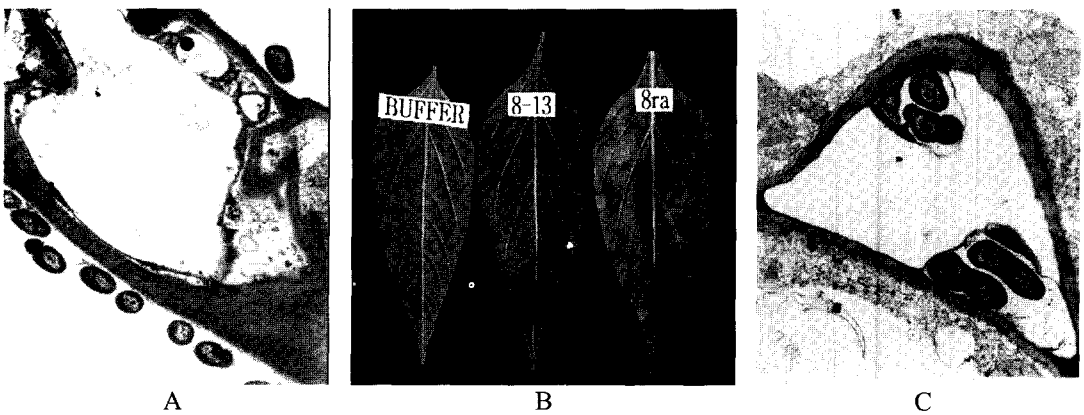

Fig. 1. Symptoms (**B**) and electron micrographs (A and C) of hot pepper plant following infiltration of soybean pustule pathogens *Xanthomonas campestris* pv. *glycines* 8ra (**C**) and 8-13 (**A**). Photos and electron micrographs were taken 24 h after syringe infiltration.

of differentially expressed hot pepper cDNA clones were isolated following inoculation with *Xcg*. DNA sequencing analysis revealed that some of them were similar to known genes as induced by pathogen but others were identified as unknown function(s) through sequence similarity search. About 60% of isolated genes had unknown functions while the remaining had significant sequence homology to already known proteins (Lee et al., unpublished data). The four functional categories (metabolism, stress and defense, protein synthesis and destination, and signal transduction) covered 31% of the isolated genes. The rest of isolated genes were composed of energy, transcription, cellular organization, and channel/transporter related genes (Lee et al., unpublished data).

Northern blot analyses show that most of the selected genes were up regulated under both HR+ and HR- conditions but not by buffer infiltration (Lee et al., unpublished data). In addition, most genes show differential transcriptional activity in resistance and susceptible reactions of pepper against its natural pathogen, *Xanthomonas campestris* pv. *vesicatoria* (Lee et al., unpublished data). To get a detailed expression profiling, hundreds of clones were arrayed on a slide glass called cDNA microarray (Schena et al., 1995). The microarray experiments are being done to monitor the regulation patterns during different stresses giving indication on the roles of each gene during plant resistance processing.

Random EST Sequencing and Data Analysis

After *in vivo* excision of a pathogen-induced hot pepper cDNA library, thousands of recombinant plasmids were isolated from individual single colonies for DNA sequencing. Single pass 5'-end sequencings were determined using the dye terminator sequencing method (Applied Biosystems, USA). Vector and unclear parts of the obtained DNA sequences were clipped using Phred software and all the sequences were then constructed as DB of FASTA format (Ewing and Green, 1998). Clustering and removing of redundant sequences of the FASTA sequence DB were performed using Phred/Phrap contig-assembly algorithm (Fig. 2). After removing the redundant sequences, the unique DNA sequences were filtered through Local BLAST multi-process and classified based on the functional categories of the genes. Computational analysis and functional classification of EST sequence data obtained in this study is being pursued.

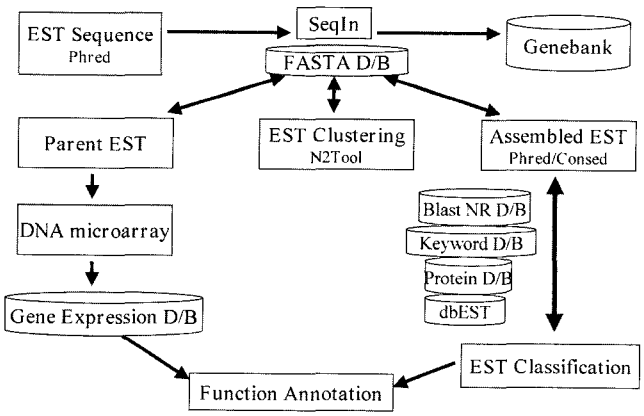

Fig. 2. Flow chart of clustering, BLAST analyses, cDNA microarray, and classification of randomly sequenced EST clones from pathogen-induced hot pepper cDNA library.

Identification of Gene Function via Functional Genomics Tools

A large number of hot pepper genes, either related to pathogen or not, was isolated. The next step was the isolation of genes related to plant resistance processes and identification of the function(s) of selected genes. To achieve the goals of this study, expression patterns of each gene were monitored to determine the function(s) by either using *in silico* Northern blots or experimental Northern blots. To monitor expression patterns, cDNA micro-array is a good functional genomic tool (Schena et al., 1995). Micro-array experiments provide a good parallel analysis of expression of genes (Kazan et al., 2001; Lee, 2001). Furthermore, micro-array can reveal the possible stimuli that regulate gene expression (Kazan et al., 2001). It can be used as a powerful tool to determine gene function identification (Brown and Botstein, 1999; Kazan et al., 2001). The annotated information should be confirmed experimentally. The *in vivo* confirmation of gene function can be carried out in various ways. Because of the low rate of transformation in hot pepper, the gene silencing will be applied as an alternative method (Baulcombe, 1996; Zhu et al., 1996). The successful application of virus induced gene silencing (VIGS) into hot pepper plant could accelerate gene function identification that would likely be used for high-throughput gene function analysis.

Conclusion

This study aimed to isolate and characterize the function of a complete set of genes induced in hot pepper plant during resistance against pathogen. The overall procedure for functional genomic approaches is shown in Fig. 2. As an initial stage, thousands of genes related to the expression of disease resistance in pepper were isolated. Although computational tools for analysis of massively isolated cDNA sequences were developed, the tools are being improved

to get more accurate results. Studies on the functional genomics of isolated hot pepper genes in relation to defense against pathogen using DNA micro-array and VIGS methods will be performed along with massive EST data.

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